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Review

Brown midrib mutations and their importance to the utilization of maize, sorghum, and pearl millet lignocellulosic tissues

Scott E. Sattler [a,b], Deanna L. Funnell-Harris [a,c], Jeffrey F. Pedersen [a,b,*]

[a] Grain, Forage and Bioenergy Research Unit, USDA-ARS, Lincoln, NE 68583-0737, United States
[b] Department of Agronomy and Horticulture, University of Nebraska-Lincoln, Lincoln, NE 68583-0737, United States
[c] Department of Plant Pathology, University of Nebraska-Lincoln, Lincoln, NE 68583-0737, United States

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ABSTRACT

Brown midrib mutants have been isolated in maize (*Zea mays*), sorghum (*Sorghum bicolor*) and pearl millet (*Pennisetum glaucum*) arising by either spontaneous or chemical mutagenesis. The characteristic brown coloration of the leaf mid veins is associated with reduced lignin content and altered lignin composition, traits useful to improve forage digestibility for livestock. Brown midrib phenotype is correlated with two homologous loci in maize (*bm1* and *bm3*) and sorghum (*bmr6* and *bmr12*), which encode cinnamyl alcohol dehydrogenase (CAD) and a caffeic O-methyl transferase (COMT). These enzymes are involved in the last two steps of monolignol biosynthesis. In maize, *bm* phenotype is associated with increased livestock digestibility, but at the cost of significantly reduced forage and grain yields. In sorghum, yield reductions were apparent in near isogenic lines, but were ameliorated through construction of hybrids that maintain reduced lignin content and increased digestibility. Near-isogenic sorghum brown midrib lines and hybrids are dispelling old beliefs that brown midrib mutants are significantly more susceptible to plant pathogen attack and to lodging than their non-brown midrib counterparts. Brown midrib mutants from new chemically mutagenized populations hold promise of identifying a non-redundant set of genes involved in lignification of grasses. In addition, early reports indicate brown midrib mutants significantly increase conversion rate in the lignocellulosic bioenergy process.

Contents

* Corresponding author at: Grain, Forage and Bioenergy Research Unit, USDA-ARS, University of Nebraska-Lincoln, Lincoln, NE 68583-0737, United States. Tel.: +1 402 472 1754; fax: +1 402 472 4020.
E-mail address: Jeff.Pedersen@ars.usda.gov (J.F. Pedersen).

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1. Introduction

This review of the brown midrib literature will build on previously published reviews and focus primarily on research results published since a thorough 1991 review of the brown midrib literature [1]. Hypotheses and dogma regarding the value of brown midrib mutations have changed substantially in some areas since that time due to refinement of research and the utilization of materials isogenic for brown midrib genes. For example, our 2005 review on reduced lignin and its impact on plant fitness [2] concluded that reduction of lignin in crop plants negatively impacts agricultural fitness. This review focusing on brown midrib mutations reports new information leading to the conclusion: using heterosis (hybrid vigor), agricultural fitness as well as end-use quality can be enhanced.

2. Brown midrib phenotype

2.1. Occurrence

The first documented spontaneous occurring brown midrib phenotype in maize (*Zea mays*) was observed over eighty years ago [3]. The characteristic reddish-brown to tan colored midribs of mutant leaf blades contrasts with the pale green midrib of wild-type leaf blades. Mutant plants also accumulated reddish-brown to yellow pigment in stalks and roots. This phenotype has been associated with reduced lignin levels and altered lignin composition compared to wild-type for over forty years [4]. Since its identification in maize, the brown midrib mutants have been isolated in two other C4 grasses, sorghum (*Sorghum bicolor*) [5] and pearl millet (*Pennisetum glaucum*) [6]. In these cases, chemical mutagens (diethyl sulfate or ethyl methane sulfonate) were used to induce mutations in these grasses and brown midrib mutants were isolated in subsequent generations [5–7]. Brown midrib mutants have not been reported in other C4 species such as sugarcane (*Saccharum* spp.) or switchgrass (*Panicum virgatum*) probably due to genetic redundancy in their polyploid genomes.

Interestingly, brown midrib mutants have not been identified or described as such within the C3 grasses. Genetic redundancy in polyploid C3 grasses could explain the absence of brown midrib mutants in wheat (*Triticum aestivum*) or oats (*Avena sativa*), but rice (*Oryza sativa*), rye (*Secale cereale*) and barley (*Hordeum vulgare*) are all diploid grasses like maize, sorghum and pearl millet. The lack of the "brown midrib" mutants in rice or barley, which have fairly extensive mutant stocks, suggests that the phenotype presents itself differently in C3 grasses. The cloning and characterization of the rice *GOLD HULL AND INTERNODE2* (*GH2*) locus, which encodes a cinnamyl alcohol dehydrogenase (CAD2) involved in lignin biosynthesis, supports this view [8]. The midribs of *gh2* leaves do not accumulate the brown coloration, although this mutant is defective in a gene orthologous to brown midrib mutants in both maize and sorghum [9]. A phenotypically similar mutant, *brown culm*, has been isolated in rye, which has been described as having light-brown/orange coloration of the stems (nodes and internodes) and spikes (rachis, glumes and awns) [10]. However, it remains to be determined whether *gold hull and internode1*, *3*, *4* (rice) or *brown culm* (rye) affect lignin biosynthesis. For reasons yet to be determined, C3 grasses do not accumulate the characteristic light-brown pigment in the midribs of their leaf blades, perhaps due to biochemical and anatomical differences between C3 and C4 grass leaves.

2.2. The brown pigment associated with altered lignin biosynthesis

Why do the brown midrib mutants accumulate the reddish brown to tan pigment in midribs and stalks? Unfortunately, there are no clear answers to this question nor has the chemical composition of the pigment been determined. Initial investigations recognized that pigmentation in brown midrib mutants was localized to lignified tissues where it was inextractable [3]. Early biochemical analyses indicated that the brown pigmentation was not due to accumulation of carotenoids, anthocyanins, flavones, tannins or flavonols [3]. Interestingly, abnormal reddish-brown coloration of lignified tissues has been observed across vascular plants (from dicots to gymnosperm) when monolignol biosynthesis has been impaired either by mutation or antisense/RNAi technologies [11–16]. These results indicate that the altered coloration of lignified tissue, which results from disruption of the monolignol biosynthesis pathway at several different steps, is not specific to any particular group of vascular plants or any step in the pathway [11–16]. In addition, a particular change in lignin composition cannot be readily associated with brown pigmentation, because these mutants and transgenic lines are impaired in monolignol biosynthesis at different steps of the pathway, which cause dissimilar compositional changes to the lignin polymer [11–16]. Mutants or transgenic lines with impaired cinnamyl alcohol dehydrogenase (CAD) activity are exceptions in which the reddish coloration of lignified tissue has been attributed to the incorporation of cinnamyl aldehydes into lignin in place of cinnamyl alcohols [12,13,15,17]. For mutants and transgenic lines impaired in other steps in monolignol biosynthesis, it has been suggested that altered coloration is due to incorporation of phenolic compounds other than coumaryl, coniferyl or sinapyl subunits into lignin. While the exact cause of the change in coloration is elusive, it appears to be a good marker for impaired monolignol biosynthesis in C4 grasses.

3. Characterization and cloning *brown midrib* loci

Only five brown midrib (*bm1* through *bm5*) loci have been identified to date in maize, an extensively studied and genetically characterized plant. The *bm1*–*bm4* are spontaneous mutants that were first isolated and characterized decades ago [18]. Recently, a fifth locus, *bm5* was identified [19]. *bm5* and *bm2* are represented by single alleles and *bm4* by two alleles in the Maize Genetics and Genomics Database (MaizeGDB) [20], suggesting saturation for brown midrib mutants has not been achieved. Efforts to intensively screen chemically mutagenized populations for the brown midrib phenotype in maize have not been reported.

Four sorghum brown midrib loci (*bmr2*, *bmr6*, *bmr12* and *bmr19*) have been identified [21]. 28 *bmr* mutants were isolated from a diethyl sulfate mutagenized population in the 1970s [5] and additional *bmr* mutants recently have been isolated from an ethyl methane sulfonate (EMS) mutagenized population [7,22]. Allelic

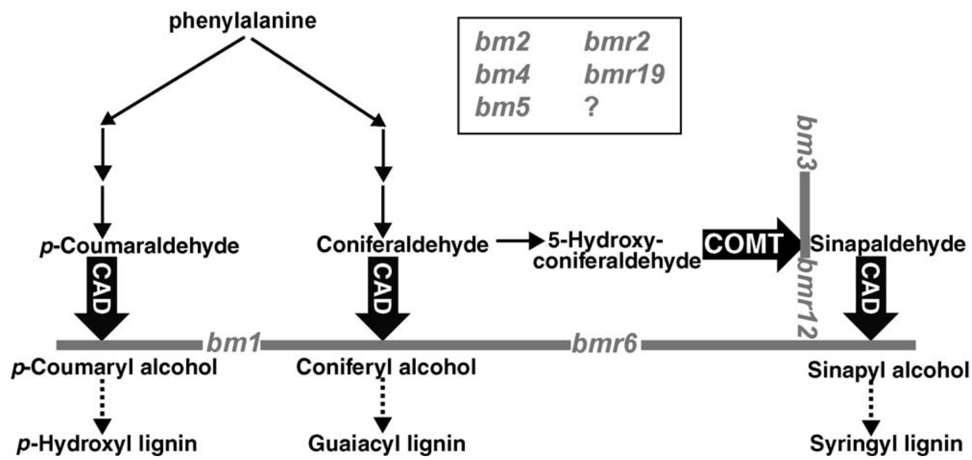

Fig. 1. The impact of brown midrib mutants on COMT and CAD enzymes in monolignol biosynthetic pathway. A simplified model of the lignin biosynthetic pathway where COMT catalyzes the addition of a methyl group to 5-OH-conferyl alcohol and CAD catalyzes the reduction of cinnamyl (coumaryl, coniferyl and sinapyl) aldehydes to alcohols in the final step of monolignol biosynthesis. *bm3* and *bmr12* impair COMT activity, and *bm1* and *bmr6* impair CAD activity.

relationships of most of the original 28 and subsequent additional mutants has not been published. In spite of the large numbers of brown midrib mutants originally isolated from sorghum, a single allele represents the *Bmr19* locus at this time [21]. This may indicate that either genetic saturation has not been achieved or alternatively, *bmr19* is not a simple loss of function mutation.

Only three pearl millet brown midrib mutants have been described in the literature; the first mutant came from a diethyl sulfate mutagenized population [6], the second was a spontaneous mutant from germplasm isolated in Zimbabwe [23], and the third mutant, arose spontaneously in Tifton, GA [24]. The three mutants are allelic [23,24]. Consequently, pearl millet is relatively uncharacterized in terms of brown midrib loci. In coming years, it is likely that additional brown midrib loci will be identified in C4 grasses.

To date, the genes corresponding to four brown midrib loci have been identified, two in maize (*Bm1* and *Bm3*) and two in sorghum (*Bmr6* and *Bmr12*) (Fig. 1). They represent two pairs of orthologous loci in maize and sorghum.

3.1. Bm3 and Bmr12

Maize *Bm3* and sorghum *Bmr12* loci have been cloned and encode orthologous caffeic *O*-methyltransferases (COMT) [25–27]. Bm3 and Bmr12 are members of an evolutionarily conserved O-methyltransferase family, whose function in lignin biosynthesis has been documented in both monocots and dicots [11,28–30]. In the penultimate step of monolignol biosynthesis, COMT transfers a methyl group from *S*-adenosyl-methionine (SAM) to the 5-hydroxyl group of 5-hydroxy-coniferyl substrates to form sinapyl products (Fig. 1). The lignin monomeric composition of *bm3* and *bmr12* plants has shown that syringyl-lignin was greatly reduced, while p-hydroxyphenyl- and guaiacyl-lignin were slightly reduced [31–33]. In addition, a novel lignin monomer, 5-hydroxy guaiacyl, was significantly elevated in *bm3* and *bmr12*, which resulted from the reduction of COMT activity and the subsequent accumulation of 5-hydroxy coniferyl alcohol [31–33].

Three maize *bm3* alleles have been isolated and the mutation sites identified. *bm3-1* contains an insertion of an LTR retrotransposon in the second exon, and *bm3-2* and *-3* contain large deletions within the second exon [26,34]. All three are presumably null alleles, because the deletions or insertion occur in open reading frames prior to sequence encoding the SAM binding site. The COMT protein was not detected using 2D gel electrophoresis in *bm3-1* protein extracts [35], supporting this position.

Six distinct alleles of sorghum *bmr12* have been isolated and the mutated sites identified [7,21,25]. Nonsense mutations are responsible for four of the characterized alleles [7,21,25], and two alleles are caused by missense mutants [7,21]. The four nonsense mutations are all presumably null alleles, because the premature stop codons would truncate the polypeptide prior to the SAM binding site of the enzyme [21,25]. In addition, Bmr12 protein was not detected in the *bmr12-ref* protein extracts using immunoblot analysis, supporting this view [32]. The two missense mutations change evolutionarily conserved amino acids to structurally dissimilar amino acids, P150L and G335S [7,21], however these amino acids occur outside the active site or known motifs within COMT. The effects of these missense alleles on either enzymatic activity or lignin composition have not been published, however for the P150L allele midrib coloration, stalk coloration and lignin staining of stalks were intermediate relative to WT and G335S allele [7]. These results suggest bmr12 P150L retains some COMT activity. The *bm3* and *bmr12* alleles that have been extensively studied should completely the lack the activity of corresponding COMT gene products, because they contain nonsense mutations for the reasons discussed above. However, COMT activity was still detectable in *bm3* plants, although it was reduced to 10% of wild-type activity [36]. This result suggests that Bm3 is the major protein responsible for COMT activity, and that other O-methyltransferases present in maize can utilize cinnamyl substrates.

3.2. Bm1 and Bmr6

Bmr6 in sorghum encodes a cinnamyl alcohol dehydrogenase 2 (CAD2) [9,37]. The *Bm1* locus was previously mapped to the orthologous *ZmCAD2* gene in maize, but a mutation was not identified [38]. In the final step of monolignol biosynthesis, CAD catalyzes the reduction of cinnamyl aldehydes (coniferyl, coumaryl and sinapyl aldehyde) to their corresponding cinnamyl alcohols, using NADPH as a cofactor, prior to their incorporation into the lignin polymer (Fig. 1). All H-, G- and S-subunits were significantly reduced in *bm1* and *bmr6*, and coniferyl and sinapyl aldehydes were incorporated in the lignin polymer in place of their corresponding alcohols at detectable levels [18,21,31,33,39,40]. Similar results have been observed in other CAD2-deficient plants [12,13,15,17].

The CAD2 family is evolutionarily conserved across vascular plants, and mutants or transgenic repression (antisense or RNAi) lines have demonstrated its essential function in monolignol synthesis in several species including loblolly pine, tobacco, alfalfa, *Arabidopsis*, rice, and poplar in addition to maize and sorghum [8,12,13,15,17,41,42]. The mutations responsible for *bm1* have not been identified, and protein immunoblot analysis indicated that ZmCAD2 protein was significantly reduced from *bm1* protein extracts using a polyclonal antibody raised against the tobacco CAD2 protein [38]. ZmCAD2 is an ortholog to both the sorghum Bmr6 and rice Gh2, mutations in either gene resulted in reduced CAD activity and altered lignin composition similar to the *bm1* phenotype. Together these results suggest that the *Bm1* locus encodes the maize ZmCAD2. The mutations in three *bmr6* alleles have been identified. The nonsense mutation in *bmr6-ref* (Gln132 to STOP) truncates the reading frame prior to the NADPH binding and C-terminal catalytic domains [9,37]. The Bmr6 protein was not detected by protein immunoblot in *bmr6-ref* extracts [9]. Together these data indicate that *bmr6-ref* is a null allele. There is a missense mutation (G191S) in *bmr6-3* and a frameshift resulting in the truncation of the last 27 amino acids in *bmr6-27* [37]; interestingly, both alleles are phenotypically comparable to *bmr6-ref* [21,37]. Although CAD2 protein was absent from *bmr6* tissues, CAD activity was still detectable in these tissues, though activity was reduced to 15–50% of wild-type activity [38–40]. These results indicate that there are other CAD proteins present in sorghum that can utilize cinnamyl substrates, but the brown midrib phenotype reveals that *Bmr6* encode the main CAD protein in the monolignol biosynthetic pathway in sorghum.

Examination of the CAD2 amino acid sequences showed that nearly all of the critical amino acids are conserved between grasses and dicots with the exception of amino acid 57 near the active site; in grasses there is a histidine at that position instead of an asparate or glutamate found in other vascular plants (dicots, gymnosperm and lycophytes) [9]. Based on the crystal structure of the orthologous CAD from *Arabidopsis* (AtCAD5), the proposed catalytic mechanism involves hydride transfer from NADPH to the aldehyde substrate coordinated by the catalytic zinc in the active site of the enzyme. Both Thr49 and His52 are critical to this process and participate in the proper orientation of the cofactor and in the hydride transfer [43], and both amino acids are present in Bm1 and Bmr6 [9]. The change from the ancestral acidic amino acid, Asp or Glu, to the basic amino acid His might have some functional significance to catalytic activity, and/or substrate specificity. Unlike dicots, grass cell walls contain significant amounts of ester linked *p*-coumaric acids and both ester and ether linked ferulic acids that are separate from lignin polymers [44,45]. A majority of *p*-coumarate is esterified to sinapyl alcohol prior to its incorporation into cell walls [46,47]. This amino change might be an adaptation involved in the unique phenylpropanoid requirements for grass cell wall formation. Use of plant transformation and site-directed mutagenesis may provide insight into the significance of this amino acid change and whether it has broader effects on the enzymatic activity and phenylpropanoid metabolism in grasses.

3.3. Bm2, Bm4, Bmr2 and Bmr19

Although neither the *Bm2* nor the *Bm4* locus has been cloned, both loci have been genetically mapped to chromosomes 1 and 9, respectively (MaizeGDB; http://www.maizegdb.org/) [20]. A map position for *bmr2* has not been published, but both *bmr2* and *bm2* have phenotypically similar effects on lignin composition: H-lignin is unaffected, G-lignin is greatly reduced, and S-lignin is increased or unchanged relative to wild-type [21,31,33,48,49]. In addition, *bm2* plants did not accumulate any novel subunits unlike *bm3*/*bmr12* and *bm1*/*bmr6* [31,33,48,49]. The *bm2*/*bmr2* phenotype is the converse of the *bm3*/*bmr12* phenotype where H- and G-lignin are relatively unaffected and S-lignin is greatly reduced, which led to the suggestion that Bm2 is a regulatory protein involved in limiting the flux from coniferyl substrates to sinapyl substrate [49]. This hypothesis suggests that ferulate 5-hydroxylase and COMT enzymes might be ectopically or temporally over-expressed in *bm2*, which would result in an increase of S-subunits with a parallel reduction of G-subunits, but this hypothesis has not been tested. In *bm4*, only modest changes in lignin composition were observed relative to wild-type, and no unusual lignin subunits were present at elevated levels, [31,33], unlike *bm3*/*bmr12* and *bm1*/*bmr6*. The impact of *bm4* on lignin biosynthesis remains an open question. In *bmr19*, lignin content determined by the Klason method was not significantly reduced compared to wild-type, but *bmr19* lignin composition showed a reduction in G-subunits, which was not as dramatic as *bmr2* [21].

4. Expression patterns in *bm* mutants

Recently, gene expression in *bm* mutants has been examined using array-based methods. One group used a macro-array consisting of gene-specific tags based on 651 maize cell wall related ESTs [50], while the other group utilized subtractive suppressive hybridization (SSH) and the maize unigene microarray (http://www.plantgenomics.iastate.edu/maizechip/) [51]. Of the 651 cell wall related sequences, 144 were expressed in 20-day-old maize stems and 69 genes were expressed in at least one of the *bm* mutants (1–4) [52]. *bm1* and *bm2* had the greatest numbers of differentially expressed genes, 55 and 47, respectively while *bm3* had the fewest number of differentially expressed genes, 7 [52]. Interesting, all the differentially expressed genes were decreased relative to the isogenic wild-type line of *bm1*, and similarly all except two differentially expressed genes were decreased in *bm2* [52]. The down-regulated genes from *bm1* included five CAD genes including *ZmCAD2* as well as other genes related to phenylpropanoid metabolism and several cell wall-related transcription factors [52]. These data have led to the speculation that *bm1* and *bm2* might be transcriptional regulators or regulatory proteins [52]. As previously mentioned *bm1* has not been cloned, but it has been mapped to a locus containing the *ZmCAD2* gene. Not unexpectedly, the expression data between the two groups showed little similarity, due to different gene sets represented by each platform, plant stages, and isogenic backgrounds [51,52]. Fifty-three ESTs were differentially expressed across three isogenic lines for *bm3* [51], consistent with the macro-array data that indicated *bm3* had little overall effect on gene expression. Thirty-two ESTs were differentially expressed in all three mutants (*bm1*, *bm2* and *bm3*) [51]. Approximately 70% of the genes identified by SSH were not present on the unigene microarray, which indicates, along with other data, that it represents about 30% of the maize genome [51]. Together, the SSH and the unigene datasets also indicated that several CAD genes were down-regulated in *bm1* [51]. It is difficult to explain how mutating a single *CAD* gene, *ZmCAD2*, could affect the mRNA levels of several other CAD genes. Expression of phenylpropanoid related genes were down-regulated in the *bm1*, *2* and *3* except for the CYP98A1 gene, a phenolic hydroxylase [51]. However, 5–7-week-old basal and ear internodes from plants at the silking stage expressed several phenylpropanoid related genes as increased levels in *bm3* relative to wild-type [53], but it is difficult to surmise the degree of overlap between genes represented on the macro-array and those represented on the unigene microarray. Together these data may underline the plasticity of lignin biosynthesis, which is influenced by plant stage, tissue position and genetic background. These data also illustrate the need for common platforms representing the entire transcriptome. The function of most of the ESTs in phenylpropanoid metabolism has been assigned solely based on sequence similarity to experimentally documented proteins from other organisms, so there are multiple genes assigned to each step. The sound conclusion is that the *bm* mutants affect the expression of genes related to phenylpropanoid metabolism, but the biological relevance of changes in gene expression are unclear at this point.

5. Performance, composition and utilization

5.1. Maize

5.1.1. bm3

The maize *bm3* mutation has been incorporated into commercial hybrids and brown midrib corn was recently reported in the popular press to represent about 5% of the silage market in Canada [54]. Brown midrib maize is generally viewed as being lower yielding than non-brown midrib maize but contributing to increased production when fed to lactating dairy cows due to its reduced lignin content and increased digestibility. The representative comparative nutrient composition of a *bm3* maize hybrid and its isogenic non-brown midrib control hybrid is shown in Table 1.

The average grain yields of the brown midrib lines were reduced by 20%, and average stover yields were reduced by 17% in experiments using a set of fifteen *bm3* lines and their 15 normal isogenic lines [56]. Reduction in dry matter yield (15–20%) in *bm3* isolines compared to their normal counterparts [57] and in 21 hybrids compared to their *bm3* isoline counterparts [58] was also reported. Still, recent maize breeding efforts have resulted in commercially available brown midrib hybrids. However, some reduction in dry matter yield usually remains associated with the brown midrib phenotype. In the 2008 Wisconsin corn hybrid performance trials [59], one brown midrib hybrid had dry matter yield equivalent to the trial mean. Mean yield of five other brown midrib hybrids exhibited a 13% lower dry matter yield than trial means at various locations.

bm3 maize is clearly targeted for silage used in dairy production. The direct effect of *bm3* in maize as discussed above is reduction of lignin content. Possibly the most centrally held view, that *bm3* maize is associated with increased fiber digestion resulting in increased dry matter intake, higher energy intake, and increased milk yield, was confirmed using isogenic *bm3* and non-brown midrib maize hybrids [60]. These results using high-producing dairy cows are, however, far from universal. Using maize hybrids of unknown genetic similarity, no difference was found in dry matter intake associated with the brown midrib trait, but milk yield was higher for cows fed the brown midrib hybrid [61]. Another group found increased dry matter intake, but no effect of *bm3* on milk production [62]. The underlying effect of *bm3* in maize is reduced lignin content, which remains consistent throughout the literature. The impact of *bm3* on dairy production is complex, and very likely associated with stage of lactation, level of productivity [60,62] and diet formulation [55].

Table 1
Less lignin and more digestible materials in brown midrib maize hybrids[a].

	Near-isogenic control (g/kg)	bm3
Neutral detergent fiber (cellulose + hemicellulose + lignin)	429	414*
Acid detergent fiber (cellulose + lignin)	224	202*
Lignin	20	13*
Crude protein	77	75
Ash	39	36*
Starch	354	383*
In vitro true dry matter digestibility	782	833*
In vitro neutral detergent fiber digestibility	465	559*

[a] Adapted from Oba and Allen (2000).
* bm3 and control differ significantly at $P < 0.05$.

5.1.2. bm1, bm2, bm4

Little has been published describing the effects of *bm1*, *bm2*, and *bm4* on the agronomic performance of maize. The *bm1* mutation appears to decrease days to flowering while the *bm2* mutation appears to increase days to flowering [63]. A single study reports no reduction in dry matter yields in *bm1* maize hybrids [64]. A search of the scientific literature failed to identify any differences in animal performance when fed *bm1*, *bm2*, or *bm4* maize.

5.2. Sorghum

Although it is generally believed that the effect of sorghum brown midrib mutations on yield is similar to those reported in maize [65], few comparisons of yield of brown midrib sorghum and their isogenic wild-type counterparts have been published in the scientific literature. The results of extensive yield trials [66–68] support the hypothesis that brown midrib sorghums are generally associated with lower yields. Brown midrib sorghum hybrids averaged 12% less than non-brown midrib hybrids over three years. However, in these same yield trials some individual brown midrib sorghums were among the highest yielding hybrids indicating that in agricultural practice, performance should be evaluated in terms of hybrids being considered by producers, and that for basic science, effects of brown midrib genes would best be considered within isogenic genetic backgrounds. Neither brown midrib genes nor genetic relationships of hybrids were identified in the above yield trials.

As with maize, brown midrib mutations in sorghum generally lower lignin content, resulting in increased fiber digestion with concomitant increased dry matter intake, higher energy intake, and increased animal performance [1]. However unlike maize, the sorghum industry developed and deployed brown midrib hybrids utilizing genes differing in mechanism of lignin reduction. Since the classical 1991 review, most sorghum brown midrib utilization research has involved multiple genetic backgrounds and/or comparisons of *bmr6* which decreases CAD activity, and *bmr12* (or its allele *bmr18*) which decrease COMT activity. The development and release of lines isogenic for *bmr6*, *bmr12* and wild-type in multiple genetic backgrounds [69–71] has greatly facilitated this line of research.

5.2.1. bmr6 vs. wild-type in two genetic backgrounds

A multi-state forage trial comparing two wild-type and near-isogenic *bmr6* and wild-type sudangrass [*S. bicolor* subsp. Drummondii] varieties [72] the effect of *bmr6* on yield was influenced by both environment and cultivar. Yields of the *bmr6* isoline of one variety were not always reduced, while yields of the *bmr6* isoline of the other variety were reduced in both locations as compared with their wild-type counterparts. Conversely, brown midrib isolines were higher in all measures of forage nutritional value than their wild-type counterparts and the effect of the *bmr6* gene on nutritional traits was generally greater in one variety leading to the conclusion that linkage or epistatic interactions of *bmr6* and quantitative trait loci associated with differences in the wild-type lines are probably responsible for the differential effect of *bmr6*.

5.2.2. bmr6 vs. bmr12 (or bmr18) vs. wild-type

The first research report comparing the effects of different brown midrib genes in sorghum on animal performance [73] showed that *bmr6* sorghum silage contributed to higher milk yields when fed to dairy cows than a wild-type sorghum silage diet. Dairy performance of the *bmr18* sorghum silage diets did not differ significantly from either the *bmr6* or wild-type sorghum silage diets (Table 2). Nutrient composition data of the silage and apparent total tract digestibility of the balanced diets for this

Table 2
Higher digestibility and dairy performance of cattle consuming brown midrib forage sorghum silage[a].

	Wild-type	*bmr6*	*bmr18*
Sorghum silage[b]			
Neutral detergent fiber (g/kg) (cellulose + hemicellulose + lignin)	581	502	482
Acid detergent fiber (g/kg) (cellulose + lignin)	377	336	285
Acid detergent lignin (g/kg)	29	23	25
Crude protein (g/kg)	73	75	78
Ash (g/kg)	41	45	33
Starch (g/kg)	109	145	168
Total tract digestibility (of balanced ration)			
Dry matter (g/kg)	525[b]	629[a]	691[a]
Neutral detergent fiber (g/kg)	408[c]	544[a]	479[b]
Lactational performance (of balanced ration)			
Milk (kg/d)	31.0[b]	34.1[a]	32.2[ab]
Milk fat (%)	3.57[b]	3.89[a]	3.77[ab]
4% fat corrected milk (kg/d)	29.1[b]	33.7[a]	31.2[ab]

Means within a row with different superscripts differ ($P < 0.05$).
[a] Adapted from Oliver et al. (2004).
[b] No tests for statistical differences provided in original manuscript.

experiment are also shown in Table 2. The only statistically significant differences ($P \leq 0.05$) between the *bmr6* and bmr18 silages were for apparent total tract NDF digestion with *bmr6* silage being 14% higher than the bmr18 silage, and subtle differences in ruminal acetate to propionate ratios [73]. The treatment means appear to support the conclusion that not all bmr hybrids will elicit similar digestibility and performance responses, and the authors correctly point out that the effects of the specific mutations are confounded with hybrid in this study weakening the already tentative conclusion. This study and the associated table are included in this review because to our knowledge they represent the only known published comparative data involving utilization of *bmr18*.

Much more extensive research on the comparative effects of *bmr6* and *bmr12* were subsequently published by the same group utilizing lines isogenic for these two genes [69–71]. Averaged across four forage sorghum genetic backgrounds, wild-type had higher average yields than *bmr12* isolines, which had higher average yields than *bmr6* isolines [74] (Table 3). However, in one genetic background, *bmr6* yields were equivalent to wild-type, and in a different genetic background *bmr12* yields were equivalent to wild-type. Acid detergent lignin (ADL) was usually highest in wild-type and lowest in *bmr12* lines. Overall in vitro neutral detergent fiber (IVNDF) means showed both *bmr6* and *bmr12* to be superior to wild-type lines for fiber digestion, but no differences in IVNDF were shown within two sets of individual isolines as compared with near-isogenic wild-type lines. Gene effects consistent across four genetic backgrounds included days to anthesis, with *bmr12* lines averaging 4 days later maturity than wild-type, and height, with wild-type being consistently tallest.

In a companion grain sorghum paper, average wild-type grain yields were higher than average *bmr6* grain yields, which were higher than average *bmr12* grain yields [75] (Table 3). Residue yields following grain harvest were highest for *bmr12* lines, possible due to later maturity. Mean residue ADL was lowest for *bmr12* lines, intermediate for *bmr6* lines, and highest for wild-type lines. Mean IVNDF was highest for *bmr12* lines, intermediate for *bmr6* lines, and lowest for wild-type lines. In the only animal feeding experiment published using these isolines to date, beef cattle gains when grazing residue of a *bmr12* grain sorghum hybrid were doubled compared to those grazing the wild-type isohybrid [76]. Grain yields of the isohybrids were equivalent.

The subsequent release and description of a subset of lines with stacked *bmr6* and *bmr12* (both mutant genes in the homozygous condition) isogenic to their wild-type, *bmr6*, and *bmr12* lines discussed above [77] once again demonstrated the importance of genetic background on the effect of bmr genes. In a forage sorghum background, the stacked isoline had reduced yield compared to either the *bmr6* or *bmr12* isoline, which had reduced yields when compared to the wild-type. ADL was considerably lower in the stacked isoline compared to the single *bmr* gene isolines which were lower than the wild-type line. In two grain sorghum stacked isolines, grain yield and residue yield, and residue ADL of the stacked isolines relative to their *bmr6*, *bmr12* and wild-type counterparts was highly influenced by background.

A common question from end-users and individuals starting new brown midrib breeding efforts is "which gene is better?" To end users, the answer is very clear: "it depends on the interaction of the *bmr* gene and the genetic background." Comparison of *bmr6* and *bmr12* in multiple genetic backgrounds indicates that the effects of individual brown midrib genes within specific sorghum lines–and extrapolating, within hybrids - are not uniform. End-users of brown midrib sorghum should always evaluate the performance of specific hybrid/gene combinations. It is certainly possible to produce superior brown midrib hybrids containing either the *bmr6* or *bmr12* mutation. For new breeding efforts the answer may depend upon the growth stage at which brown midrib materials are utilized. Our data is based on laboratory and animal trials utilizing mature sorghum and clearly suggest that on average, *bmr12* is more effective at reducing lignin and increasing fiber digestibility. Other labs focusing on end-use at a vegetative stage have emphasized *bmr6*. Some basis for choice of *bmr* gene may lay in the fact that *bmr6* impairs CAD activity and, consequently, reduces all three forms of lignin, while *bmr12* affects COMT activity primarily resulting in reduction in S-lignin [32], which increases with advancing maturity [78].

5.3. Pearl millet

The three reported brown midrib mutants in pearl millet represent a single locus, but the enzyme affected by these mutations has not been reported. The brown midrib trait is associated with significant yield reduction. A 23% reduction in yield was found on an individual plant basis when grown in spaced plots [24]. In sown field plots using near-isogenic lines of brown midrib and wild-type pearl

Table 3
Changes in agronomic performance and composition associated with brown midrib forage and grain sorghum[a].

	Wild-type	*bmr6*	*bmr12*
Forage sorghum (mean of four genetic backgrounds)			
Days to anthesis	74[b]	75[b]	78[a]
Height (cm)	215[c]	194[c]	211[b]
Lodging (%)	23	23	22
Dry matter yield (t/ha)	15.0[a]	12.8[c]	13.5[b]
Neutral detergent fiber (g/kg)	454	449	463
Acid detergent fiber (g/kg)	269[a]	262[b]	268[a]
Acid detergent lignin (g/kg)	70[a]	67[a]	61[b]
In vitro neutral detergent fiber (g/kg)	646[b]	666[a]	655[a]
Grain sorghum (mean of four genetic backgrounds)			
Days to anthesis	71[c]	72[b]	75[a]
Height (cm)	123[a]	112[b]	124[a]
Grain yield (kg/ha)	6149[a]	5135[b]	4948[c]
Residue yield (kg/ha)	5883[b]	5284[c]	6503[a]
Residue neutral detergent fiber (g/kg)	616[a]	611[b]	610[b]
Residue acid detergent fiber (g/kg)	379[a]	365[b]	354[c]
Residue acid detergent lignin (g/kg)	91[a]	77[b]	67[c]
Residue in vitro neutral detergent fiber (g/kg)	505[c]	526[b]	556[a]

Means within a row with different superscripts differ ($P < 0.05$).
[a] Adapted from Oliver et al. (2005a,b).

Table 4
Decreased yield and improved nutrient composition of brown midrib pearl millet[a].

	Wild-type	Brown midrib
Dry matter yield (t/ha)	6.6	3.8*
Neutral detergent fiber (g/kg)	649	617*
Acid detergent fiber (g/kg)	366	367
Acid detergent lignin (g/kg)	20	15*
Crude protein (g/kg)	189	216*
Ash (g/kg)	114	112*
Effective ruminal degradability		
Dry matter (g/kg)	506	573*
Neutral detergent fiber (g/kg)	275	342*

[a] Adapted from Mustafa et al, 2004.
* Means within a row with differ ($P < 0.05$).

Table 5
Genetic background of sorghum affects lodging more than brown midrib[a].

	Wild-type	*bmr6*	*bmr12*
Atlas (%lodged)	36	36	36
Early Hegari-Sart (%lodged)	7	7	7
Kansas Collier (%lodged)	18	18	19
Rox Orange (%lodged)	29	30	29

[a] Adapted from Oliver et al. (2005a).

millet a 42% reduction in first harvest yield associated with the brown midrib trait was reported (Table 4), but there was no reduction in second harvest yields [79]. As in maize and sorghum, ADL was significantly reduced in brown midrib isolines and digestibility of dry matter and fiber was significantly increased (Table 4). We speculate that the very significant yield reductions associated with the brown midrib trait in these and other studies may be attributable in part to the relative lack of breeding applied to incorporation of this recently induced [6] or discovered [23,24] mutations into pearl millet compared to brown midrib maize and sorghum. Characterization of existing mutations and subsequent discovery of additional brown midrib mutations with alternate modes of action (e.g. reduction of COMT vs. reduction of CAD) may provide breeders with better strategies to incorporate brown midrib into pearl millet lines without the severe associated yield loss reported to date.

6. Future directions

6.1. Lodging susceptibility

Nearly a century of dogma has relegated brown midrib crops to a market niche where improved nutritional quality traits add adequate value to overcome associated deficiencies including reduced yield, increased lodging, and increased disease susceptibility. Regarding yield, the recent near-isogenic comparisons in maize, sorghum, and pearl millet discussed support the hypothesis that brown midrib mutations are generally associated with reduced yield. However, our recent work demonstrated that grain yields equivalent to wild-type, and enhanced residue yields can be obtained in specific sorghum hybrids [75] indicating that yield reductions previously associated with brown midrib mutations can be overcome. Furthermore, in formal yield trials comparing commercial hybrid forage sorghums, individual brown midrib hybrids were among the higher yielding hybrids in each of the past three years [66–68]. With focused applied plant breeding, continued improvements in yield are anticipated in brown midrib hybrids for all three crop species.

Lodging of brown midrib maize is generally assumed. A higher incidence of stalk breakage at maturity in brown midrib maize compared with normal lines [80] and a 17–26% decrease in crushing strength in three *bm3* hybrids compared to their normal counterparts was described [81]. However, an increase in lodging attributable to brown midrib was not detected in several other maize studies, possibly due to overriding effects of genetic backgrounds [57,82]. Results are inconsistent in yield trials involving commercial hybrids. No lodging [66], higher average lodging [68] and lower average lodging in brown midrib hybrids [67] have been reported in the same location during the course of three years.

The genetic background in which brown midrib genes are deployed is critically important regarding lodging. Results of replicated field studies with *bmr* genes deployed in isogenic sorghum lines showed obvious line effects, but no significant differences attributable to *bmr* genes [74] (Table 5). Reduction in actual or perceived lodging associated with brown midrib crops should be attainable.

6.2. Disease susceptibility

The assumption that brown midrib plants are inherently more disease susceptible, is also being challenged. Lignin provides a physical barrier against initial attack [83,84] and lignin or lignin-like phenolic polymers are induced and rapidly deposited in cell walls in response to both biotic and abiotic stresses, which may prevent further growth or confine invading pathogens [83,85–88]. However, perturbations of the lignin biosynthetic pathway may cause accumulations of lignin precursors, and many of these precursors have been shown to inhibit growth of pathogenic fungi or inhibit production of virulence factors [89–92]. For example, accumulation of ferulic acid, *p*-coumaric acid, and sinapic acid has been correlated with resistance to *Fusarium* spp. [91,93]. Although not specifically associated with brown midrib maize, a recent patent documents modification of a lignin biosynthetic pathway enzyme, cinnamate 4-hydoxylase, causing reduced lignin content, increased digestibility, and increased resistance to *Fusarium moniliforme* in maize [94].

Brown midrib sorghum is associated with reduced infection by members of *F. moniliforme* [95]. When comparing grain from lines isogenic for *bmr6*, *bmr12*, and wild-type, *bmr12* plants had significantly fewer colonizations by *F. moniliforme* (which includes the sorghum pathogen *Fusarium thapsinum*) and both bmr lines had fewer colonizations by other *Fusarium* spp. When peduncles were inoculated with a *F. thapsinum* isolate, lesions resulting on brown midrib lines were significantly smaller than those on wild-type lines (Fig. 2). More complete understanding of brown midrib mutations, and complete saturation of maize, sorghum, and pearl millet genomes, will undoubtedly lead to further hypotheses regarding the impact of brown midrib genes on plants and on plant responses to pathogens.

6.3. Bioenergy

Beyond the use of brown midrib mutants to increase forage digestibility, there has been significant interest in the impact potential these mutants may have on lignocellulosic bioenergy. Lignocellulosic bioenergy conversion requires decomposition of the cell wall polysaccharides cellulose and hemicellulose into monomeric sugars prior to their conversion into ethanol or alternative biofuels. Lignin negatively impacts lignocellulosic conversion because it can block the enzymatic liberation of sugars from cell wall polysaccharide moieties, releases aromatic compounds that can inhibit microbes used for fermenting sugars to fuels, and adheres to hydrolytic enzymes. Therefore brown midrib feedstocks, which have reduced lignin content and altered lignin composition, would likely have increased conversion efficiency over their wild-type counterparts. However, publications on this subject are currently very limited. The enzymatic saccharification efficiency (conversion of cell wall polysarcharides to their sugar

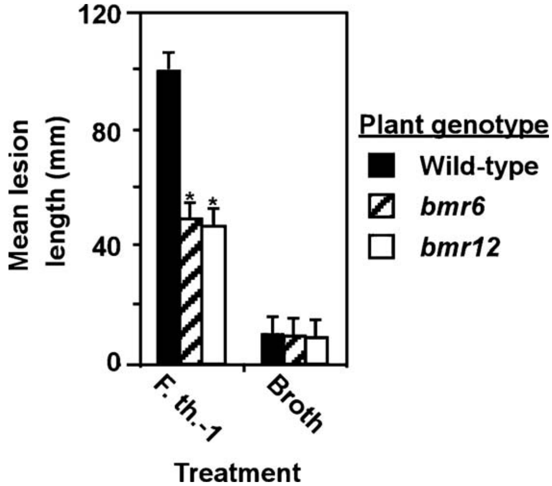

Fig. 2. Mean lesion lengths resulting from inoculation of peduncles of wild-type and near-isogenic *bmr6* and *bmr12* plants with the sorghum pathogen *F. thapsinum* (formerly known as *F. moniliforme*). Two weeks following anthesis, plants were wound inoculated with toothpicks incubated in broth cultures of *F. thapsinum* or sterile broth (control). Eighteen days following inoculation, peduncles were split longitudinally and length of the resulting lesion was measured. Positive standard errors are shown. "*" indicates that mean lesion length is significantly less than that of wild-type within the same treatment ($P \leq 0.05$).

monomers using hydrolytic enzymes) of sorghum *bmr2*, *bmr6* and *bmr12* stover was increased by up to 17%, 20% and 21%, respectively, relative to wild-type [21]. Similarly, A brown midrib forage sorghum stover had highest hexose yield (79% for maximum) following enzymatic hydrolysis as compared to non-bmr stover that yielded 43% and 48% of this maximum [96]. However, neither the brown midrib mutants nor the genetic background were described in this publication [96]. A *bmr6* and *bmr12* forage sorghum stover had higher hexose yield (79% and 77% for maximum, respectively) following enzymatic hydrolysis compared to wild-type stover that yielded 65% of the maximum while the highest hexose yield (90% of maximum) was observed in *bmr6 bmr12* double mutant stover [97]. The reduced lignin in *bmr6*, *bmr12* and the *bmr6 bmr12* double mutant stovers increased ethanol conversion efficiency (44%, 46%, 57%, respectively) compared to wild-type (38%) [87]. Within this isogenic forage sorghum background, lignin (Klason) content had a strong negative correlation with ethanol conversion efficiency ($r = -0.943$). Together these studies establish that brown midrib mutants can increase hexose yield in enzymatic saccharification, which will translate into higher ethanol conversion efficiencies. In particular, it was [97] confirmed that lignin is a major factor negatively affecting the lignocellulose to ethanol conversion process. Stacking *bmr* mutants translated into additive effects in terms of ethanol conversion [97]. Combining different brown midrib genes may be a promising research direction to reduce lignin content and increase conversion efficiency both for livestock and bioenergy. Potentially, the use of *bmr* mutants could reduce the severity of the pretreatment through reducing the amount of caustic chemicals required, the duration of the pretreatment or the heat required, which could have wide range benefits including reducing the cost of process or increasing the efficiency through a reduction in the monomeric sugar degradation during the pretreatment.

6.4. Brown midrib mutants compared to impairing monolignol biosynthesis through transgenics

Many of the genes involved in monolignol biosynthesis have been transgenically down-regulated resulting in reduced lignin content and alter lignin composition in a range of dicotyledonous plants [11–13,16]. Similarly, transgenic approaches to insert glycoside hydrolases, modify cellulose synthesis and crystallinity, modify hemicellulose and pectin, and integrate water-soluble polymers within the cell walls of plant systems have been recently reviewed [98]. Although these strategies have been effective in experimental settings, they have not been as well utilized in maize, sorghum or pearl millet, probably because of three main reasons. First, it has been relatively easy to obtain brown midrib mutants from chemically mutagenized populations. Second, unlike some transgenic lines, these mutations are stable. Third, open release of transgenic sorghum is currently restricted world-wide, whereas the brown midrib mutants have been released as commercial products in both maize and sorghum. A major advantage that brown midrib mutants have over transgenic strategies is that the deployment of brown midrib mutants does not involve the costly regulatory hurdles that antisense/RNAi lines require.

Similar to transgenic down-regulation, chemical mutagenesis has the potent to generate mutations, which result in a range of partial losses of function. Examples include missense *bmr12* alleles [7]. Unlike antisense/RNAi approach, chemical mutagenesis also can generate mutations causing the complete loss of gene product function, examples include the nonsense alleles of *bm3*, *bmr12* and *bmr6* [9,21,25,26,34,35,37]. TILLING has also led to the prospect of isolating mutations in a target gene in both maize and sorghum [7,21]. However, as indicated within this article, a majority of studies and breeding efforts have utilized the alleles of *bm3*, *bmr12* and *bmr6* that contain nonsense mutations. Although these nonsense alleles likely completely block the activity of the gene product, CAD (*bmr6*) or COMT (*bm3* and *bmr12*) [9,25–27,35,37], there are still residual CAD or COMT activities present in the mutant plants [31–33,36,40]. Lignin compositional analysis also indicated S-lignin was still present in *bm3* and *bmr12* tissues, and H-, G- and S-lignin were still present in *bmr6*. Together these data demonstrate that while *Bm3*, *Bmr12*, and *Bmr6* encode the main COMT and CAD enzymes, respectively there are other o-methyl transferase and alcohol dehydrogenase genes within both maize and sorghum that prevent complete blockage of either step in monolignol biosynthesis in the null alleles of *bm3*, *bmr12* and *bmr6*.

In maize, there is one opportunity to directly compare a brown midrib mutant, *bm3* to COMT antisense lines; COMT enzymatic activity was less severely impaired in the two antisense lines compared to *bm3*, which led to less severe reduction in S-lignin content [98]. Lignin content as determined by Klason lignin was similar between the antisense lines and *bm3* [99]. A clear advantage in plant fitness between COMT antisense lines and *bm3* was not reported [99]. However, this study did highlight the possibility of using tissue specific promoters to impair the monolignol biosynthesis in specific tissues or cell-types [99].

7. Conclusion

We are on the cusp of major change in dogma regarding brown midrib plants. Research and understanding of "brown midrib" is rapidly moving from a phenotypic trait with an associated reduction in lignin, to the identification of a series of well-defined genes with differing gene-function. This process will be enhanced by the discovery of new brown midrib loci through use of chemical mutagenesis nearing saturation for the phenotype [7,22]. These screens should define the number of non-redundant gene products involved in lignification of C4 grasses and provide new resources for breeding and for basic research to support growing livestock and emerging bioenergy markets for products with enhanced lignocellulosic chemical profiles.

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